

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application No.	: 10/679,951	Confirmation No.: 3589
Applicant	: LUTHER, et al.	
Filed	: October 6, 2003	
TC/AU.	: 1646	
Examiner	: Zachary C. Howard	
Docket No.	: 2923-571	
Customer No.	: 6449	<u>May 16, 2007</u>

RULE 132 DECLARATION OF DR. BERND MÜHLENWEG

I, Dr. Bernd Mühlenweg, declare as follows:

1. I am the same Dr. Bernd Mühlenweg named as one of the inventors in the above-referenced application.

2. My educational background and experience are as shown on my *curriculum vitae*, which is attached hereto as Exhibit A.

3. I am familiar with the subject patent application, including the currently pending claims. I am also familiar with the Office Action mailed September 21, 2006, and the subsequent Advisory Action, in which the Examiner has maintained a rejection of claims 39-42, 45-49, 56-59, and 61. The Examiner has stated that the specification is “enabling for a method of providing a prognosis for a breast-cancer patient comprising measuring the ratio of uPAR del4+5 deletion variant to del2+3+4” but “does not reasonably provide enablement for a (1) a [sic] method for the detection of tumors associated with generation of uPAR deletion variants; (2) a method of predicting the risk of cancer in a tumor-bearing patient; (3) or a method of providing prognosis for a tumor-bearing patient.” As I understand, it is therefore the Examiner’s position, as stated in the Advisory Action, that “the specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with” the rejected claims.

4. I make this Declaration in order to make of record certain data relating to the subject matter of the present claims. The data was first presented in an Amendment filed February 6, 2007 in connection with the subject application. The Examiner has requested that the data now be presented in the form of a Declaration submitted under 37 C.F.R. §1.132.

5. The following experiments were conducted under a grant in form of a company - university cooperation. I have been the project manager for this cooperation on behalf of the company and have therefore been involved in the planning and interpretation of the experiments and results.

6. The prognostic relevance of the mRNA concentration of the uPAR-mRNA variants uPAR-del4/5 and uPAR-del5 was analyzed in a representative sample of 43 breast cancer primary tumors. In this connection, highly sensitive quantitative RT-PCR assays were established. The results showed that uPAR-del4/5 is expressed with a high frequency (97%) and independently from the uPAR wild-type. Increased concentrations of uPAR-del4/5-mRNA in the primary tumor were statistically highly significant ($p=0.0004$) and associated with an increased relapse rate and a poor prognosis of breast cancer patients (Luther et al., Thromb Haemost 2003; 89: 705-17).

7. **Results:**

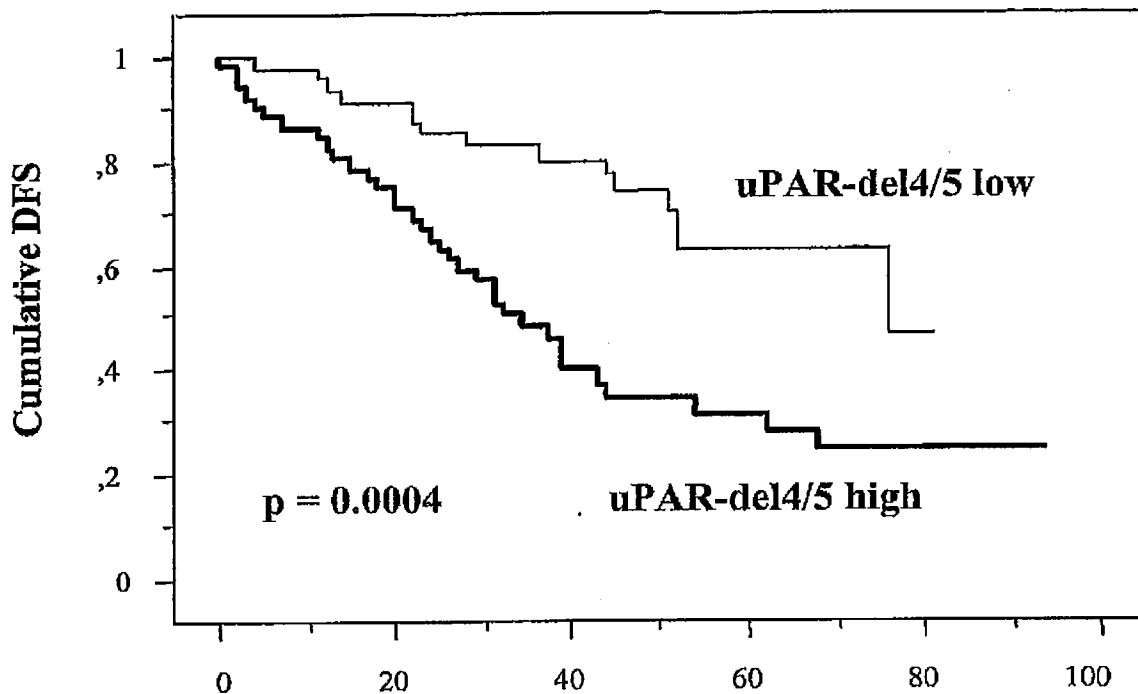
1) Expression of uPAR-del4/5-mRNA in breast cancer tissues

102 tumor extracts were analyzed in order to validate the prognostic significance of uPAR-del4/5-mRNA in breast cancer. For this purpose, the wild-type uPAR-2/3/4-mRNA and the uPAR-del4/5-mRNA concentrations were determined and normalized in relation to the reference gene GAPDH using the methods described in the publication noted in paragraph 6 above. The lymph node status, the BR grading and the uPA, as well as PAI-1 concentrations in the elected representative sample were statistically significantly associated with the relapse-free survival of breast cancer patients. It could also be shown in this larger

study that uPAR-del4/5 is expressed in breast cancer tissue with a high frequency (88%) and independently from the wild-type uPAR-mRNA ($r=0.23$, n.s.). In addition, increased concentrations of uPAR-del4/5-mRNA in the primary tumor were statistically highly significantly ($p=0.0004$) associated with an increased relapse rate and a poor prognosis of breast cancer patients, whereas wild-type uPAR-mRNA and wild-type uPAR antigen concentrations (determined by means of ADI Imubind ELISA) did not show a statistically relevant relation for prognosing the tumor disease.

Figure 1: Significance of the uPAR-del4/5-mRNA concentration in the primary tumor

The probability of a relapse-free survival (DFS) in a sample of patients with primary breast cancer was calculated using the Kaplan-Meier statistic.

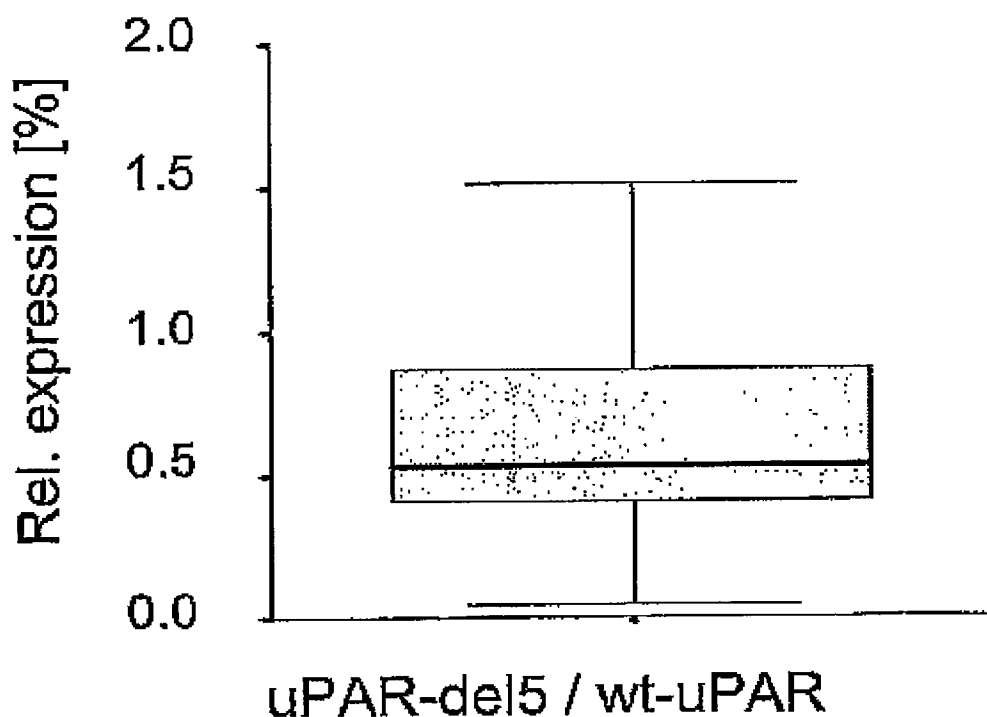


2) Development of a RT-PCR assay for the detection of uPAR-del5-mRNA

A specific, highly-sensitive quantitative RT-PCR assay was established for the detection of the uPAR-del5-mRNA (see Enclosure 1: Farthmann et al.). A further RT-PCR

assay was additionally established for the detection of wild-type uPAR-2/3/4/5-mRNA with comparable transcript size.

Figure 2: Relative uPAR expression in relation to corresponding wild-type uPAR-2/3/4/5- mRNA expression in tissue extracts obtained from primary breast cancer

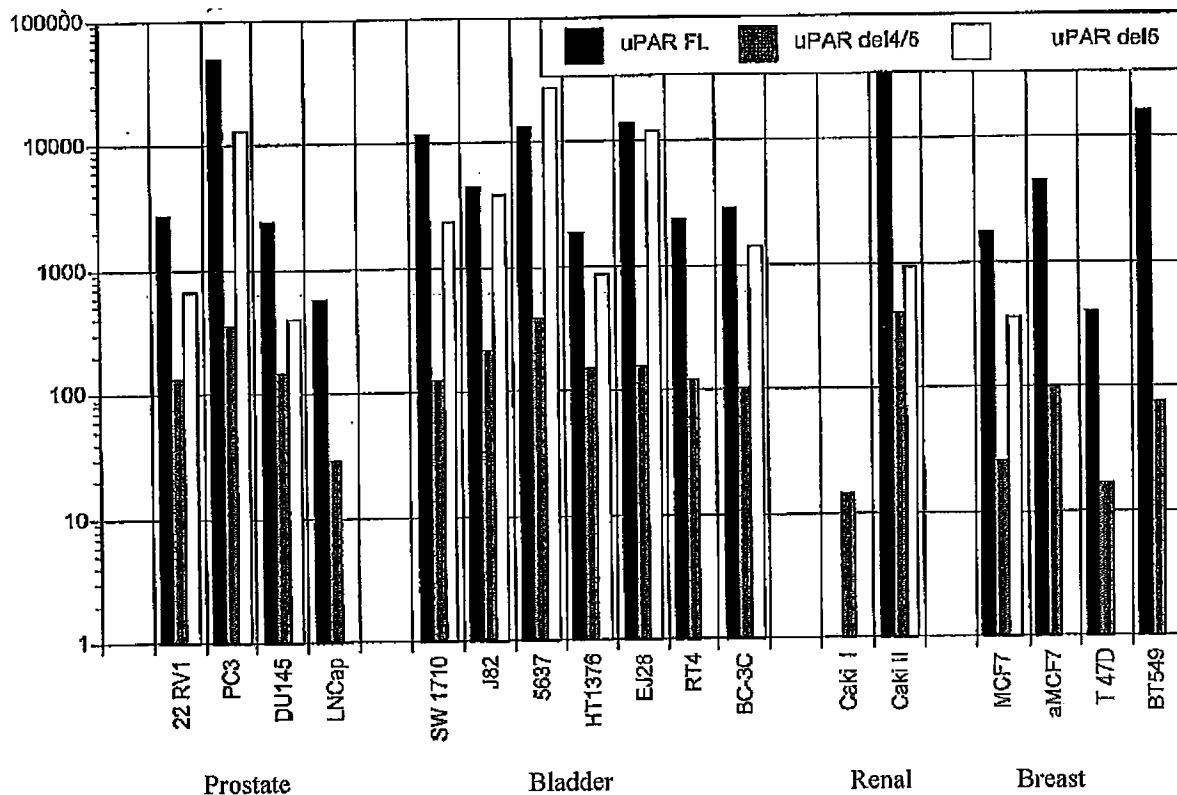


For validation of the two PCR assays, tumor cell lines and breast cancer tissue from the above-described sample (from approximately 40 samples, since cDNA was not sufficiently available from all of the tumor tissues) as well as from a new sample of 174 tumor samples (see Farthmann et al.) were analyzed. For this purpose, the uPAR-2/3/4/5-mRNA and uPAR-del5-mRNA concentrations were determined and normalized in relation to the reference gene G6PDH using the methods described in the above-mentioned publication. The uPAR-del5-mRNA could be detected in the analyzed sample with a high frequency, but with a relatively low level of expression (approximately 1% of the wild-type RNA, Fig. 2). In the analyzed sample, a correlation of the wild-type uPAR-mRNA and uPAR-del5- mRNA ($r=0.779$, $p<0.001$) could be detected.

3) Expression analyses of uPAR-del4/5 and -del5 in further carcinoma cell lines

The results of expression analyses on an available panel of well characterized ATCC cell lines of urological carcinomas demonstrated the presence of splice variants in all tested tumor cell lines of epithelial origin (Fig. 3). In accordance with the findings gained by means of the breast carcinoma tissues, a universally and generally dominant expression of the wild-type uPAR-mRNA compared to the deletion variant can be expected. The uPAR-del5-mRNA expression could not be detected in some of the cell lines, as a rule more uPAR-del5 transcripts than uPAR-del4/5 transcripts were detectable.

Figure 3: Relative expression (detected molecules in the approach for measuring) of wild-type uPAR-2/3/4 (=uPAR FL), uPAR-del4/5 and uPAR-del5-mRNA in tumor cell lines



y-coordinate: Relative Expression (Molecules)

x-coordinate: Prostate carcinoma; Bladder carcinoma; Renal carcinoma; Breast carcinoma

All in all, cDNA samples of four prostate carcinoma (PCa) cell lines, 7 bladder carcinoma (BCa) cell lines and 2 kidney cell carcinoma cell lines were analyzed in comparison to 4 breast cancer cell lines.

8. In sum, it can be stated that the transcript variants of the uPAR deletion mutants del4/5 and del5 were detectable in human cell lines of the three most common urological carcinoma entities.

9. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: May 16, 2007

Signed: Bernd Mühlenweg
Dr. Bernd Mühlenweg

RESUME

Name	Dr. Bernd Mühlenweg
Address	Hippmannstr. 28, D-80639 Munich, phone: +49 (89) 51 72 78 95, email: bernd.muehlenweg@web.de
Date of Birth	September 28, 1971
Citizenship	German
Marital Status	Married, 3 children

EMPLOYMENT

Since 03/2007	Senior Manager Business Development, Wilex AG, Munich, Germany
03/2004–02/2007	Manager Business Development, Wilex AG
02/2002–02/2004	Associate Business Development, Wilex AG
04/2001–01/2002	Junior-Scientist and Group Leader “Assay Development and New Products”, Wilex AG
01/2000–03/2001	Scientist and Group Leader “Somatic Gene Therapy”, Department of Gynecology, Technical University of Munich, Germany

EDUCATION

02/2000	Final examination <i>PhD</i> , Department of Chemistry and Medical School, Technical University of Munich
03/1997–02/2000	<i>PhD</i> studies, Medical School, Department of Gynecology, Technical University of Munich
12/1996	Final examination Diploma of Chemistry, Department of Biochemistry, University of Bielefeld, Germany
04/1995–07/1995	Research project, Faculty of Chemistry, Dublin City University, Ireland
11/1991–12/1996	Undergraduate studies, Faculty of Chemistry, University of Bielefeld

CIVIL SERVICES

1989–2000	Voluntary fire brigade (Bielefeld, Gilching/Munich), several advanced training courses, e.g. operation control and leading of troops
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EXPERIENCE

License Agreements (exclusive, non-exclusive, cross-licensing)

Patent purchase agreements

Commercialization agreements (research antibodies)

Due diligence (M&A, in-licensing)

Competitive intelligence and market analysis

Project management and out-licensing of non-core projects

Research cooperation agreements (GLP- and non-GLP-compliant, university and corporate)

Manufacturing agreements (GMP- and non-GMP-compliant)

GXP-agreements phase I to phase III (investigator/hospital agreements (16 countries), CRO agreements, insurances, central review agreements, GDP-agreements)

Research fund raising (national & international, accumulated granted volume >5.3 Mio. Euro)

ADDITIONAL QUALIFICATIONS

Languages English – fluent in spoken and written

French – basic knowledge

Software Microsoft Office and system software

MS Project

MS Access

Acrobat

Data basis ADIS R&D Insight

IDdb3

Pubmed

Training courses Leadership Program (St. Galler Business School, 10–11/2006)

M&A Workshop (KPMG USA, 06/2006)

Tactics for negotiations (Pharmaventures, 09/2002)

NPV-calculation (Pharmaventures, 09/2002)

Research marketing (Technical University of Munich, 07/2000)

According to §§ 15/17 GenTSV: Projektleiter für gentechnische Arbeiten und Beauftragter für biologische Sicherheit

AWARDS, PUBLICATIONS AND PATENTS

AWARDS

Johannes-Zange-Price 2005 of the North-German Society for Otorhinolaryngology and zervicofacial Surgery: Publication price for Ergongur et al. (see below).

PUBLICATIONS

Killeen SD, Andrews EJ, Wang JH, Wu T, Schmalix W, Muehlenweg B, Redmond HP. Inhibition of urokinase plasminogen activator with a novel enzyme inhibitor, WXC-340, ameliorates endotoxin and surgery-accelerated growth of murine metastases. *Br J Cancer* 2007, 96: 262-8.

Setyono-Han B, Stürzebecher J, Schmalix WA, Muehlenweg B, Sieuwerts AM, Timmermans M, Magdolen V, Schmitt M, Klijn JGM, Foekens JA. Suppression of rat breast cancer metastasis and reduction of primary tumor growth by the small synthetic urokinase inhibitor WX-UK1. *Thrombosis and Haemostasis* 2005, 93: 779-86.

Ertongur S, Lang S, Mack B, Wosikowski K, Muehlenweg B, Kastenbauer E, Gires O. Inhibition of the invasion capacity of carcinoma cells by WX-UK1, a novel synthetic inhibitor of the urokinase-type plasminogen activator system. *Int J Cancer*. 2004, 110: 815-24.

Diehl P, Hantke B, Hennig M, Gradinger R, Tschesche H, Mittelmeier W, Schmitt M, Muehlenweg B. Protein expression of MMP-13, uPA, and PAI-1 in pseudocapsular and interface tissue around implants of loose artificial hip joints and in osteoarthritis. *Int J Mol Med*. 2004, 13: 711-5.

Diehl P, Schmitt M, Blumelhuber G, Frey B, Van Laak S, Fischer S, Muehlenweg B, Meyer-Pittroff R, Gollwitzer H, Mittelmeier W. Induction of tumor cell death by high hydrostatic pressure as a novel supporting technique in orthopedic surgery. *Oncol Rep*. 2003, 10: 1851-5.

Hantke B, Harbeck N, Schmalfeldt B, Claes I, Hiller O, Luther MO, Welk A, Kuhn W, Schmitt M, Tschesche H, Muehlenweg B. Clinical relevance of matrix metalloproteinase-13 determined with a new highly specific and sensitive ELISA in ascitic fluid of advanced ovarian carcinoma patients. *Biol Chem*. 2003, 384: 1247-51.

Seliger B, Menig M, Lichtenfels R, Atkins D, Bukur J, Halder TM, Kersten M, Harder A, Ackermann A, Beck J, Muehlenweg B, Brenner W, Melchior S, Kellner R, Lottspeich F. Identification of markers for the selection of patients undergoing renal cell carcinoma-specific immunotherapy. *Proteomics*. 2003, 3: 979-90.

Luther T, Kotzsch M, Meye A, Langerholz T, Fussel S, Olbricht N, Albrecht S, Ockert D, Muehlenweg B, Friedrich K, Grosser M, Schmitt M, Baretton G, Magdolen V. Identification of a novel urokinase receptor splice variant and its prognostic relevance in breast cancer. *Thromb Haemost*. 2003, 89: 705-17.

Sato S, Kopitz C, Schmalix W, Muehlenweg B, Kessler H, Schmitt M, Krüger A, Magdolen V. High-affinity urokinase-derived cyclic peptides inhibiting urokinase/urokinase receptor-interaction: effects on tumor growth and spread. FEBS Lett. 2002, 528: 212-6.

Arroyo De Prada N, Schroeck F, Sinner EK, Muehlenweg B, Twellmeyer J, Sperl S, Wilhelm OG, Schmitt M, Magdolen V.
Interaction of plasminogen activator inhibitor type-1 (PAI-1) with vitronectin. Eur J Biochem. 2002, 269: 184-92.

Muehlenweg B, Sperl S, Magdolen V, Schmitt M, Harbeck N.
Interference with the urokinase plasminogen activator system: a promising therapy concept for solid tumours. Expert Opin Biol Ther. 2001, 1: 683-91.

Muehlenweg B, Assfalg-Machleidt I, Schmitt M, Macheleidt W, Magdolen V.
From monofunctional to bifunctional inhibitors: evaluation of the activity of a new generation of protease inhibitors. BIAjournal. 2001, 1: 8-11.

Muehlenweg B, Assfalg-Machleidt I, Parrado SG, Burgle M, Creutzburg S, Schmitt M, Auerswald EA, Machleidt W, Magdolen V.
A novel type of bifunctional inhibitor directed against proteolytic activity and receptor/ligand interaction. Cystatin with a urokinase receptor binding site. J Biol Chem. 2000, 275: 33562-6.

Magdolen V, Arroyo de Prada N, Sperl S, Muehlenweg B, Luther T, Wilhelm OG, Magdolen U, Graeff H, Reuning U, Schmitt M.
Natural and synthetic inhibitors of the tumor-associated serine protease urokinase-type plasminogen activator. Adv Exp Med Biol. 2000, 477: 331-41.

Muehlenweg B, Guthaus E, de Prada NA, Schmitt M, Schmiedeberg N, Kotzsch M, Creutzburg S, Kramer MD, Kessler H, Wilhelm OG, Magdolen V.
Epitope mapping of monoclonal antibodies directed to PAI-1 using PAI-1/PAI-2 chimera and PAI-1-derived synthetic peptides. Thromb Res. 2000, 98: 73-81.

Muehlenweg B, Schnelzer A, Türkmen B, Schmitt M, Lengyel E, Magdolen V.
Molecular analysis of the urokinase-type plasminogen activator gene. In: Ovarian Cancer: Methods and protocols, Bartlett JMS (Editor), 2000, Humana Press, UK.

Coles M, Diercks T, Muehlenweg B, Bartsch S, Zolzer V, Tschesche H, Kessler H.
The solution structure and dynamics of human neutrophil gelatinase-associated lipocalin. J Mol Biol. 1999, 289: 139-57.

PATENTS

Luther T, Magdolen V, Schmitt M, Kotzsch M, Muehlenweg B.
WO 02082077: Production of polyclonal monospecific antibodies against uPAR variants del4, del5, and del4+5, and the use thereof for diagnosis and therapy. Priority date: April 06, 2001.

Halder T, Harder A, Muehlenweg B, Seliger B.
DE 102 40 118.7: Verfahren zur Identifizierung von Nierenzellkarzinom spezifischen Proteinen und deren Verwertung. Priority date: August 30, 2002.

**Development of quantitative RT-PCR assays for
wild-type urokinase receptor (uPAR-wt) and its splice variant uPAR-del5 ***

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Matthias Kotzsch ³, Fred Sweep ⁴, Manfred Schmitt ¹, Paul Span ⁴, Viktor Magdolen ¹

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* a report from the Receptor and Biomarker Group of the European Organisation for Research and Treatment of Cancer

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Key words: Urokinase receptor, cancer, splice variants, mRNA, LightCycler technology

Summary

The receptor for the serine protease urokinase-type plasminogen activator, uPAR (CD 87), plays an important role in tumor cell invasion and metastasis of solid malignant tumors. uPAR is a highly glycosylated, glycan lipid-anchored membrane protein, consisting of three homologous domains. Each individual domain is encoded by two exons: DI by exons 2+3, DII by exons 4+5, and DIII by exons 6+7. Beside the wild-type (wt) uPAR mRNA, two splice variants either lacking exon 5 (uPAR-del5) or both exons 4 and 5 (uPAR-del4/5) have been described.

To specifically quantify uPAR-wt and its splice variant, uPAR-del5, we established specific, robust and highly sensitive RT-PCR assays, based on the LightCycler technology. Expression of uPAR-wt and uPAR-del5 was analyzed in different malignant cell lines as well as in a set of 174 breast cancer tissue samples. uPAR-del5 mRNA was found to be expressed very frequently at a rather low level (typically less than 1% of uPAR-wt mRNA). In tumor tissue from breast cancer patients, a statistically significant correlation between uPAR-del5 and uPAR-wt mRNA ($r = 0.779$; $P < 0.001$) was observed. There was no association between the expression level of either mRNA and clinical parameters such as nodal status, tumor size and grade. In estrogen receptor negative tumors, a significantly higher uPAR-del5 expression was found ($P = 0.023$).

The two developed quantitative RT-PCR assays described here may aid further analysis of the function and clinical relevance of uPAR-wt and one of its splice variants, uPAR-del5, in malignant tumors.

Introduction

Tumor cell dissemination and formation of metastases is facilitated by the interaction of diverse proteolytic systems, including serine proteases, cysteine proteases, and matrix metalloproteinases (Magdolen et al. 2002). These proteases enable tumor cells to degrade the extra cellular matrix and to cross natural boundaries (Sidenius and Blasi. 2003). The receptor for the serine protease urokinase-type plasminogen activator (uPAR, CD 87) is essentially involved in this process as it focuses the proteolytic activity of uPA to the cell surface. Furthermore, it interacts with a broad variety of other ligands, including vitronectin or integrins, and by this modulates proliferation, cell adhesion and migration, invasion, and angiogenesis (Blasi and Carmeliet 2002, Harbeck et al. 2004). High tumor levels of uPA and/or its inhibitor PAI-1 have been shown to be a predictor for poor prognosis of patients with solid tumors, including breast, gastric, esophageal, ovarian, colorectal or hepatocellular cancer (Reuning et al. 1998; Harbeck et al. 2004). Different therapeutic approaches have been employed to obstruct the uPA/uPAR system, using small molecules, such as antibodies or modified toxins (Muehlenweg et al. 2001, Sperl et al. 2001, Reuning et al. 2003, Liu et al. 2003).

uPAR is a highly glycosylated, glycan lipid (GPI)-anchored membrane protein, consisting of three structurally homologous domains (DI, DII, DIII) (Roldan et al. 1990). In the past, a number of glycosylation variants and different molecular forms of uPAR antigen such as soluble uPAR, uPAR-DI, and uPAR-DII+III has been described and analyzed (for a summary see Luther et al. 2003). In some cases, certain (novel) functions or activities could be assigned to these variants: endoproteolytic processing of CD87 with removal of DI is, *e.g.*, a likely pathway for controlling cell adherence and migration (Blasi and Carmeliet 2002, Montuori et al. 2002, Beaufort et al. 2004), as the extent of glycosylation of DI strongly contributes to the affinity for its ligand uPA (Møller et al. 1993). Furthermore, splice variants of uPAR have been identified, *i.e.* an uPAR mRNA splice variant lacking exon 5 (uPAR-del5) as well as a variant lacking exons 4 and 5 (uPAR-del4/5) (Casey et al. 1994, Luther et al. 2003). Since splice variants of genes often display a different expression pattern and biological role compared to that of the wild-type genes, especially in tumor tissue (Magklara et al. 2001), we previously studied the expression of the mRNA variant uPAR-del4/5 in a representative set of breast cancer tissues applying a real-time RT-PCR assay for quantification of the mRNA concentration (Luther et al.

2003). The mRNA variant uPAR-del4/5 was, in fact, expressed very frequently in breast cancer tissue and, strikingly, higher uPAR-del4/5 expression was significantly associated with shorter disease-free survival of breast cancer patients. Thus, these results suggest that uPAR-del4/5 mRNA may serve as a prognostic marker in breast cancer. The aim of the present study was to establish a highly sensitive real-time RT-PCR assay based on LightCycler technology for the uPAR-del5 mRNA variant in order to be able to analyze the expression pattern of this alternatively spliced mRNA in solid malignant tumors.

Material and Methods

Cell lines and cell lysates, uPAR ELISA assay

Human ovarian cancer cell lines OVMZ-6 and OVMZ-10, human breast cancer cell lines MDA-MB 231, MDA-MB 231 BAG, MDA-MB 435, and aMCF-7 as well as the human brain tumor cell line LN 18 were cultured at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air in DMEM medium (Gibco, Karlsruhe, Germany), supplemented with 10% fetal calf serum (Gibco) and 1% penicilline-streptomycine (Biochrom, Berlin, Germany), 1% arginine-asparagine (Sigma, Deisenhofen, Germany) and 1% HEPES buffer (Gibco). Cells were harvested from monolayer dishes after two days. Total RNA from the cells was extracted using Trizol Reagent (Invitrogen, Karlsruhe, Germany). cDNA was synthesized using AMV cDNA First Strand Synthesis Kit (Roche Diagnostics, Penzberg, Germany). cDNAs from the cell lines were diluted 1:15 and aliquoted at -20 °C.

For uPAR antigen detection, 2 x 10⁶ cells were cultured for two days on monolayer dishes, then harvested, resuspended in phosphate-buffered saline and sedimented by centrifugation (200 x g, 10 min, RT). Cells were disrupted by two freezing and thawing cycles, followed by a solubilization step (10 min, in 100 µl per 10⁶ cells sample buffer containing 0.2% Triton X-100) and stored at -20 °C. uPAR antigen was determined in cell lysates by uPAR IIF10 ELISA as described by Kotzsch and co-workers (2000). Protein content was determined using the Micro BCA protein assay kit (Pierce, Rockford, IL). uPAR antigen levels in cell lysates are expressed as ng per mg of total protein.

Patients - tissue selection

The study adhered to national regulations of The Netherlands on ethical issues and was approved by the local ethical committee. Tumor tissue was obtained from patients with unilateral breast cancer after surgical resection of the primary tumor. Patients who had received neo-adjuvant treatment, or who had a previous diagnosis of cancer or who had a carcinoma *in situ*, were excluded. Furthermore, patients with recurrent disease within one month after surgery or with distant metastases at time of diagnosis were excluded as well. After surgery, performed between November 1987 and December 1997 in participating hospitals of the Comprehensive Cancer Center East in The Netherlands, a representative part of the tumor was selected by a pathologist, frozen in liquid nitrogen, and sent to the Department of Chemical Endocrinology for routine determination of estrogen (ER) and progesterone (PgR) receptor status by ligand binding assay (Sweep and Geurts-Moespot 2000; Span et al. 2003). Remaining frozen tissue or tissue powder (after dismembration) prepared from this tumor was kept in liquid nitrogen. For the present study, samples were selected based on the availability of tissue stored in the tumor bank.

Patients- cDNA Synthesis

Total RNA was isolated from approx. 20 mg of tissue powder using the RNeasy mini kit (Qiagen, Hilden, Germany) with on-column DNase-I treatment as previously described (Span et al. 2003). Reverse transcription was performed using the Reverse Transcription System (Promega Benelux BV, Leiden, The Netherlands) according to the manufacturer's protocol. After annealing of random hexamers for 10 min at 20 °C, cDNA synthesis was performed for 60 min at 42 °C, followed by an enzyme inactivation step for 5 min at 95 °C. cDNAs were diluted 1:3 and aliquoted.

Quantitative real-time RT-PCR of uPAR-del5, uPAR-wt, and the housekeeping gene G6PDH

RT-PCR primers and hybridization probes were obtained from TibMolBiol (Berlin, Germany). RT-PCR was performed using the LightCycler apparatus (Roche, software version 3.5). Assays for quantification of uPAR-wt and uPAR-del5 were established, with primer sequences as follows: Ex 2F: GAC CTC TGC AGG ACC ACG AT; Ex 6,4R: CAG ATT TTC AAG CTC

CAG GAC TT; Ex 5A: GGT GGC GGT CAT CCT TTG. RT-PCR was performed with a master mix with 3.0 μ M MgCl₂, 0.6 μ M of the primers, 0.2 μ M of each of the hybridization probes and 2 μ l of reagent mix, in a total volume of 20 μ l. The amplification program started with pre-denaturation at 95 °C, followed by 45 cycles of amplification: denaturation 10 sec at 95 °C, annealing for 15 sec at 66 °C, and elongation at 72 °C for 15 sec. A standard curve was generated for each run using eight glass capillaries (Roboscreen, Leipzig, Germany) coated with a defined number of molecules of uPAR (wild type or del5) plasmid (range from 100,000 to 10 copies). The generation of the plasmids pRcRSV-GPI-uPAR-wt (encoding uPAR-wt) and pRcRSV-GPI-uPAR-del5 have been described previously (Luther et al. 2003). A negative control containing water as a template was included in each run. For normalization of the data h-G6PDH (human glucose-6-phosphate-dehydrogenase) Housekeeping Gene Set (Roche) was used, according to the manufacturer's protocol. All cDNA samples displaying less than 10,000 molecules of G6PDH were considered to be of non-optimal quality and were excluded from further analyses (18 of 192 cDNA samples = 9.4%). Relative expression levels were determined calculating the ratio between absolute template molecule and G6PDH housekeeping molecule numbers. The ratios of target genes and housekeeping gene were multiplied with factor 1,000.

Statistics

The association of uPAR mRNA levels with histomorphological and clinical parameters was analyzed using non-parametric tests (Mann-Whitney U test; Kruskal-Wallis test). Statistical analyses were performed using the SPSS 11.5 software.

Results and Discussion

Development of RT-PCR assays for uPAR-del5 and uPAR-wt

For quantification of uPAR-del5 mRNA, we established a highly sensitive real-time RT-PCR assay applying the LightCycler technology. In this assay, the 5' amplification primer (Ex 2F) is identical to the 5' primer used in the RT-PCR assays previously described (Luther et al. 2003)

for detection of uPAR-2/3/4 mRNA (encompassing exons 2, 3, and 4) and uPAR-del4/5 mRNA (encompassing exons 2, 3, and 6 and lacking exons 4 and 5, respectively). The 3' amplification primer (Ex 6,4R) overlaps the alternative splicing site of exons 4 and 6 and, therefore, selectively binds to uPAR-del5 mRNA (and not to uPAR-wt or uPAR-del4/5). For generation of standard curves, glass capillaries coated with defined numbers of uPAR-del5 plasmid - exactly determined by HPLC calibration (Köhler et al. 1999) - were used. In addition to the uPAR-del5 assay, a quantitative RT-PCR assay for uPAR-wt mRNA was established, which does neither amplify uPAR-del5 nor uPAR-del4/5 mRNA, since the 3' amplification primer (Ex 5A) is directed to exon 5 (Fig. 1). Since in both assays the 5' and 3' amplification primers are directed to different exons, amplification of (possible contaminating) genomic uPAR-DNA is excluded due to the large amplicon size of > 10 kbp. With both assays, the detection limit was (at least) 10 copies of cDNA, which corresponds to the lowest class capillary standard. Figure 2 depicts a plot of measured molecules *versus* theoretical molecule numbers of uPAR-del5 in 48 independent PCR runs for the standards ranging from 10 to 100,000 copies. In case of uPAR-wt, similar results were obtained (data not shown). To test for specificity of the uPAR-del5 and uPAR-wt assays, we analyzed samples containing large plasmid copy numbers (corresponding to about 1.5 pg of DNA) of either uPAR-wt, -del5, and -del4/5 or included glass capillaries coated with 100,000 copies of the three different plasmids. In neither case, we observed an amplification signal above the buffer control for the control plasmids (data not shown). Thus, the target sequence (either uPAR-del5 or uPAR-wt) is selectively amplified with the two newly established RT-PCR assays.

Quantification of uPAR mRNA variants and uPAR antigen in cell lines

Seven different cell lines were selected and the uPAR antigen content determined applying the uPAR HU/IMF10-ELISA (Kotzsch et al. 2000). As can be seen from Table 1, the uPAR antigen content ranged from undetectable (< 0.05 ng/mg, breast cancer cell line MDA-MB 435) to about 7 ng/mg total protein in the case of the ovarian cancer cell line OVMZ-10. uPAR-wt mRNA concentration (normalized to the expression of the housekeeping gene G6PDH) was determined and compared to the uPAR antigen values: the highest antigen values (OVMZ-10 and MDA-MB 231 BAG) corresponded to the highest mRNA levels, the cell line with undetectable uPAR protein levels displayed an extremely low uPAR-wt mRNA expression as well. The other cell

lines displayed an intermediate expression both at the protein and mRNA level ($r = 0.786$; $P < 0.05$) (Table 1). uPAR-del5 expression was detected in all of the cell lines albeit to a low extent ranging from 0.23 to 1.53% of uPAR-wt expression.

Quantification of uPAR mRNA variants in breast cancer tissue

We assessed the level of expression of uPAR-del5 and uPAR-wt mRNA, normalized to G6PDH mRNA, in 174 cases of breast cancer patients (Fig. 3). uPAR-wt expression was found in all cases (median relative expression level: 263). uPAR-del5 was detected in the breast cancer samples with high frequency but with a significantly lower expression level (median: 1.57). In one case, there was no uPAR-del5- amplification signal at all, in further 40 of the 174 cases (23.6%), the determined copy number was below the lowest standard included in the assay. The relative expression rate of uPAR-del5 to uPAR-wt was between 0 and 3.1% (median uPAR-del5_{rel}/uPAR-wt_{rel}: 0.53%). The mRNA concentrations of uPAR-wt and uPAR-del5 significantly correlated with each other ($r = 0.779$; $P < 0.001$). We observed no statistically significant association of uPAR-wt or uPAR-del5 expression with clinical parameters such as nodal status, tumor size or grade (Table 2). A significantly higher uPAR-del5 expression was found in estrogen receptor negative tumors ($P = 0.023$).

Conclusions

In the present study, we developed a quantitative real-time RT-PCR method to specifically quantify uPAR-wt mRNA (excluding both splice variants uPAR-del5 and uPAR-del4/5) and uPAR-del5 mRNA in breast cancer. The assays are rapid, robust, and sensitive. As these assays require only minute amounts of cDNA, they are well suited for studies when the amount of sample is limited. For validation of the uPAR-wt assay, we initially measured uPAR mRNA expression in cell lines of different origin and observed that the measured mRNA expression levels corresponded well to the respective antigen levels determined by a uPAR ELISA (HU/IIIF10). In the tumor cell lines, we also detected low expression of uPAR-del5 mRNA. *In vivo* expression of this uPAR splice variant was subsequently proven by analyzing breast tumor samples. Similar to uPAR-wt, we found no significant association of uPAR-del5 expression in relation to the nodal status, tumor size or grade in the analyzed patients cohort.

The cDNA sequence of the uPAR-del5 variant has been originally published by Casey et al. (1994). Recently, we verified expression of uPAR-del5 in tumor cells by amplification of uPAR mRNA with primers directed to exon 1 and 6, respectively, followed by direct sequencing of the resulting PCR products (Luther et al. 2003). Further evidence for the *in vivo* occurrence of uPAR-del5 comes from searching available nucleotide databases, in which a series of independent submissions of the cDNA sequence encoding uPAR-del5 obtained from various sources (*e.g.* accessions AX281707, AA481366, BM543893 or BM767461) is found. Thus, the uPAR-del5 splice variant seems to be often expressed in human cells. Previously, we have generated stably transfected Chinese hamster ovary cells, which harbor an expression plasmid encoding uPAR-del5. By ELISA, flow cytometry, and Western blot analysis, we confirmed synthesis, secretion and cell surface-association of uPAR-del5. These experiments indicate that uPAR-del5 mRNA is translated and processed in a similar manner as wild-type uPAR. Therefore, experiments are on the way to search for (new) functions of uPAR-del5 (and the other expressed splice variant uPAR-del4/5) which may play a role for tumor invasion and metastasis.

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References

- Beaufort N, Leduc D, Rousselle JC, Magdolen V, Luther T, Namane A, Chignard M, Pidard D. Proteolytic regulation of the urokinase receptor/CD87 on monocytic cells by neutrophil elastase and cathepsin G. *J Immunol* 2004; **172**: 540-9.
- Blasi F, Carmeliet P. uPAR: a versatile signaling orchestrator. *Nat Rev Mol Cell Biol* 2002; **3**: 932-43.
- Casey JR, Petranka JG, Kottra J, Fleenor DE, Rosse WF. The structure of the urokinase-type plasminogen activator receptor gene. *Blood* 1994; **84**: 1151-6.
- Harbeck N, Kates R, Gauger K, Willems A, Kiechle M, Magdolen V, Schmitt M. Urokinase-type plasminogen activator (uPA) and its inhibitor PAI-1: novel tumor-derived factors with a high prognostic and predictive impact in breast cancer. *Thromb Haemost* 2004; **91**: 450-6.
- Köhler T, Lerche D, Meye A, Weisbrich C, Wagner O. Automated analysis of nucleic acids by quantitative PCR using coated ready-to-use reaction tubes. *J Lab Med* 1999; **23**: 408-14.
- Kotzsch M, Luther T, Harbeck N, Ockert D, Lutz V, Noack F, Grossmann D, Albrecht S, Kramer MD, Lossnitzer A, Grosser M, Schmitt M, Magdolen V. New ELISA for quantitation of human urokinase receptor (CD 87) in cancer. *Int J Oncol* 2000; **17**: 827-34.
- Lin S, Aaronson H, Mitola DJ, Leppla SH, Bugge TH. Potent antitumor activity of a urokinase-activated engineered anthrax toxin. *Proc Natl Acad Sci USA* 2003; **100**: 657-62.
- Luther T, Kotzsch M, Meye A, Langerholc T, Füssel S, Olbricht N, Albrecht S, Ockert D, Muehlenweg B, Friedrich K, Grosser M, Schmitt M, Baretton G, Magdolen V. Identification of a novel urokinase receptor splice variant and its prognostic relevance in breast cancer. *Thromb Haemost* 2003; **89**: 705-17.
- Magdolen U, Krol J, Sato S, Mueller MM, Krüger A, Sperl S, Schmitt M, Magdolen V. Natural inhibitors of tumor-associated proteases. *Radiol Oncol* 2002; **36**: 131-43.
- Magklara A, Scorilas A, Katsaros D, Massobrio M, Yousef GM, Fracchioli S, Danese S, Diamandis EP. The human KLK8 (neuropsin/ovasin) gene: identification of two novel splice variants and its prognostic value in ovarian cancer. *Clin Cancer Res* 2001; **7**: 806-11.

Møller LB, Pollanen J, Rønne E, Pedersen N, Blasi F. N-linked glycosylation of the ligand-binding domain of the human urokinase receptor contributes to the affinity for its ligand. *J Biol Chem* 1993; **268**: 11152-9.

Montuori N, Carriero M, Salzano S, Rossi G, Ragno P. The cleavage of the urokinase receptor regulates its multiple functions. *J Biol Chem* 2002; **277**: 46932-9.

Muehlenweg B, Sperl S, Magdolen V, Schmitt M, Harbeck N. Interference with the urokinase plasminogen activator system: a promising therapy concept for solid tumours. *Expert Opin Biol Ther* 2001; **1**: 683-91.

Reuning U, Magdolen V, Wilhelm O, Fischer K, Lutz V, Graeff H, Schmitt M. Multifunctional potential of the plasminogen activation system in tumor invasion and metastasis. *Int J Oncol* 1998; **13**: 893-906.

Reuning U, Sperl S, Kopitz C, Kessler H, Krüger A, Schmitt M, Magdolen V. Urokinase-type plasminogen activator (uPA) and its receptor (uPAR): development of antagonists of uPA/uPAR interaction and their effects in vitro and in vivo. *Curr Pharm Des* 2003; **9**: 1529-43.

Roldan AL, Cubellis MV, Masucci MT, Behrendt N, Lund LR, Danø K, Appella E, Blasi F. Cloning and expression of the receptor for human urokinase plasminogen activator, a central molecule in cell surface, plasmin dependent proteolysis. *EMBO J* 1990; **9**: 467-74.

Sidenius N, Blasi F. The urokinase plasminogen activator system in cancer: recent advantages and implication for prognosis and therapy. *Cancer Metast Rev* 2003; **22**: 205-22.

Span PN, Tjan-Heijnen VC, Manders P, Beex LV, Sweep CG. Cyclin-E is a strong predictor of endocrine therapy failure in human breast cancer. *Oncogene* 2003; **22**: 4898-904.

Sperl S, Mueller MM, Wilhelm OG, Schmitt M, Magdolen V, Moroder L. The uPA/uPA receptor system as a target for tumor therapy. *Drug News Perspect* 2001; **14**: 401-11.

Sweep CG, Geurts-Moespot J. EORTC external quality assurance program for ER and PgR measurements; trial 1998/1999. European Organisation for Research and Treatment of Cancer. *Int J Biol Markers* 2000; **15**: 62-9.

Table 1: Detection of uPAR antigen, uPAR-wt and uPAR-del5 mRNA in seven different cancer cell lines. Cellular uPAR antigen content (expressed as ng per mg total protein) was measured using the HU/HIF10 ELISA (Kotzsch et al. 2000). uPAR-wt and uPAR-del5 mRNA was quantified by the newly established RT-PCR assays and expression normalized to G6PDH mRNA .

Cell lines		ELISA [ng/mg]	RT-PCR measurements [relative to G6PDH]		
Name	Origin	HU/HIF10	uPAR-wt	uPAR-del5	uPAR-del5 / uPAR-wt [%]
MDA 435	breast	< 0.05	21	0.2	0.95
MDA 231	breast	1.99	747	2.0	0.27
LN 18	brain	2.07	137	2.1	1.53
OVMZ-6	ovary	2.18	648	5.5	0.85
aMCF-7	breast	3.19	671	3.9	0.58
MDA 231 BAG	breast	5.28	1204	2.8	0.23
OVMZ-10	ovary	6.86	3588	28.0	0.78

Table 2: uPAR-wt and uPAR-del5 mRNA expression and clinical parameters

Associations of uPAR-wt and uPAR-del5 expression with clinical parameters were analyzed by using non-parametric tests. All tests were performed at significance level of $\alpha < 0.05$.

174 breast cancer patients			uPAR-wt / G6PDH		uPAR-del5 / G6PDH	
Variable	Total	No. of patients (%)	Mean $\pm$ SE	P value	Mean $\pm$ SE	P value
Menopausal status ^a	174					
pre/per		39 (22.4)	378 $\pm$ 53	0.397 (ns)	2.2 $\pm$ 0.3	0.785 (ns)
post		135 (77.6)	327 $\pm$ 22		2.1 $\pm$ 0.2	
Nodal status ^a	149					
negative		79 (53.0)	360 $\pm$ 31	0.058 (ns)	2.6 $\pm$ 0.3	0.070 (ns)
positive		70 (47.0)	291 $\pm$ 30		1.9 $\pm$ 0.2	
x		25				
Size (pT) ^b	171					
1		55 (32.2)	398 $\pm$ 38	0.101 (ns)	2.6 $\pm$ 0.3	0.240 (ns)
2		97 (56.7)	286 $\pm$ 20		1.9 $\pm$ 0.2	
3		15 (8.8)	405 $\pm$ 118		2.1 $\pm$ 0.4	
4		4 (2.3)	369 $\pm$ 133		1.5 $\pm$ 0.6	
x		3				
Grade ^a	123					
I/II		65 (52.8)	275 $\pm$ 23	0.116 (ns)	1.7 $\pm$ 0.2	0.112 (ns)
III		58 (47.2)	397 $\pm$ 47		2.4 $\pm$ 0.3	
x		51				
ER status ^a	174					
negative		56 (32.2)	427 $\pm$ 48	0.066 (ns)	2.8 $\pm$ 0.3	<u>0.023</u>
positive	118 (67.8)	297 $\pm$ 19	1.9 $\pm$ 0.1			
PgR status ^a	174					
negative		67 (38.5)	390 $\pm$ 39	0.274 (ns)	2.6 $\pm$ 0.3	0.153 (ns)
positive	107 (61.6)	307 $\pm$ 22	1.9 $\pm$ 0.2			
a Mann-Whitney-U Test b Kruskal-Wallis Test x Unknown status						

Figure legends

Figure 1: Detection of uPAR-wt and uPAR-del5 mRNA.

uPAR consists of three homologous domains. Each individual domain is encoded by two exons: DI by exons 2+3, DII by exons 4+5, and DIII by exons 6+7 (exon 1 encodes the signal sequence of uPAR). For specific amplification of uPAR-wt mRNA a reverse-primer directed to exon 5 was designed. In order to specifically amplify uPAR-del5 a primer overlapping the unique boundary between exons 4 and 6 was chosen. Both RT-PCR assays use the same forward primer within exon 2. The amplicon length is 376 bp for uPAR-wt and 372 bp for uPAR-del5.

Figure 2: LightCycler PCR standard curves for quantification of uPAR-del5 mRNA

The plots of molecule numbers detected *versus* theoretical molecule numbers of uPAR-del5 were generated from 48 independent PCR runs. Correlation of the values is $r = 0.990$; $P < 0.0001$).

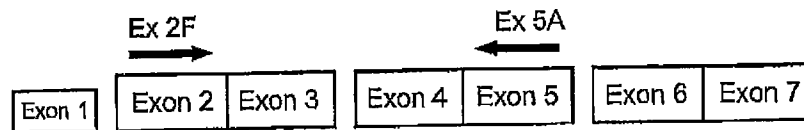
Figure 3: uPAR mRNA expression in human breast cancer tissue

174 primary breast cancer samples were measured for uPAR-wt and uPAR-del5 mRNA with the newly established quantitative RT-PCR assays. The histograms depict the frequency distribution of (A) uPAR-wt expression (normalized the housekeeping gene G6PDH), (B) uPAR-del5 expression (normalized the housekeeping gene G6PDH), and (C) relative uPAR-del5 expression compared to uPAR-wt [%].

Domains of
uPAR antigen



uPAR-wt
(376 bp)



uPAR-del5
(372 bp)

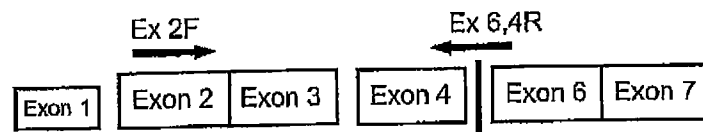


Figure 1

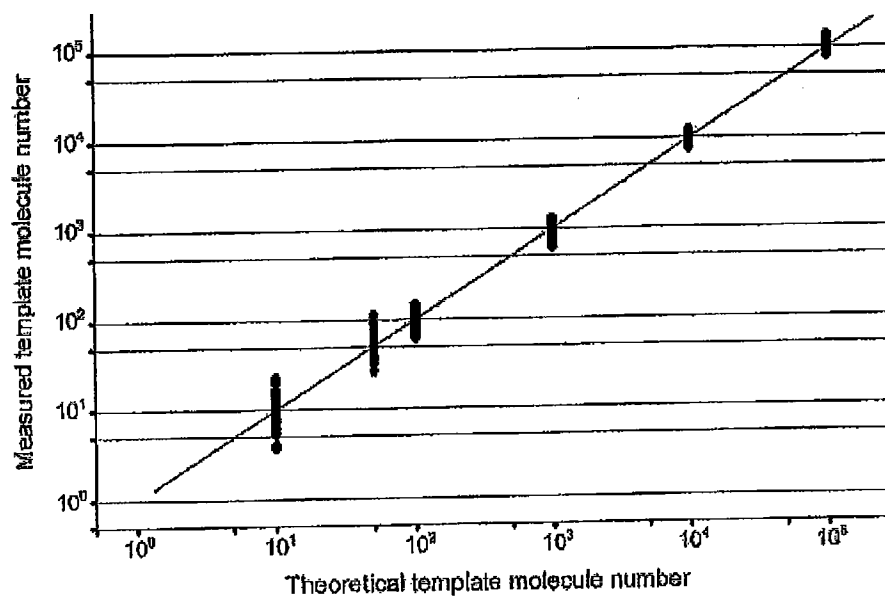


Figure 2

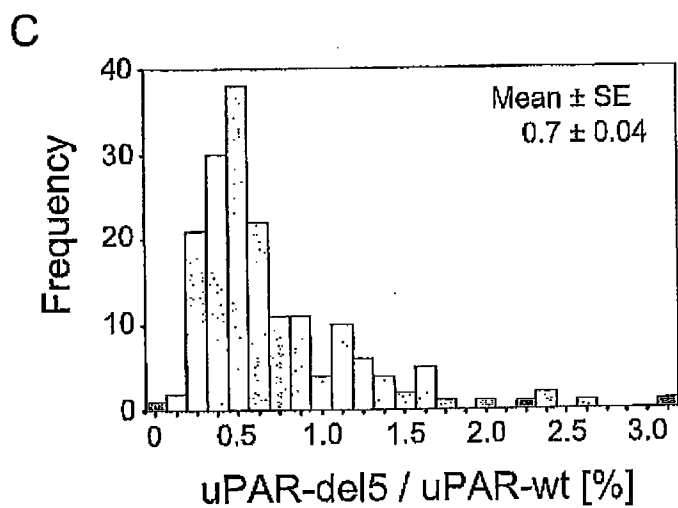
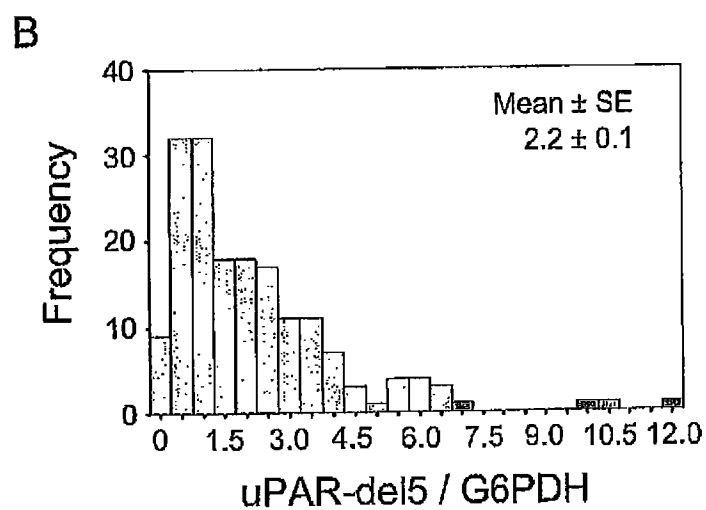
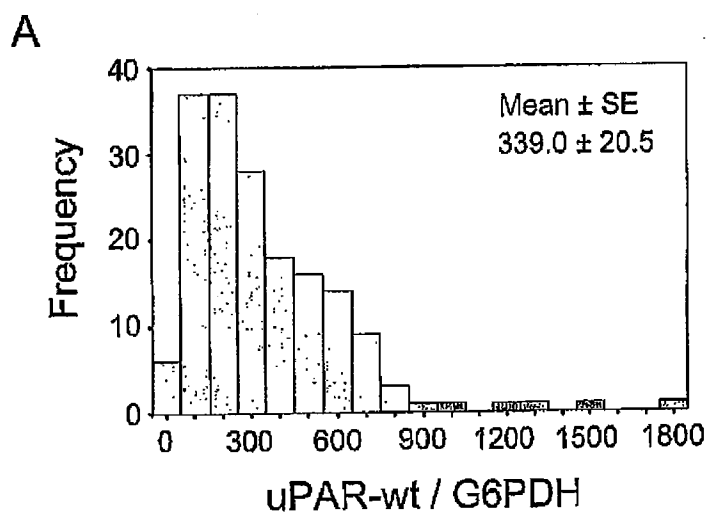


Figure 3